A comprehensive analysis of the polygalacturonase family of wheat (*Triticum aestivum* L.) shed light on important genes affecting pollen development and anther dehiscence

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Abstract

Background: Polygalacturonase (PG) belongs to a large family of hydrolases that undertake many important functions in cell separation during plant growth and development by degrading pectin. The specific expression of PG genes in pollen may have great significance for plant male sterile research and hybrid wheat breeding. However, it has not been reported in wheat (*Triticum aestivum* L.). Results: Therefore, we systematically studied the PG gene family using the latest published wheat reference genomic information. A total of 113 PGs were identified and renamed as TaPG01 - 113 based on their position on the chromosome. They were unequally distributed on 21 chromosomes and were classified into six categories of A-F. Analysis of gene structures and conserved motifs revealed that the TaPGs of Class C and D had relatively short gene sequences and a small number of introns, and Class E TaPGs were the least conserved and all members did not have III conserved domain. Segmental duplication has been shown to be one of the major drivers of the expansion of the wheat PG gene family. The cis-element predictions indicate that wheat PGs had a wide range of functions, including response to light, hypothermia, anaerobic and hormonal stimulation, and also involved in meristematic tissue expression. In addition, twelve spike-specific expressions of TaPGs were screened using RNA-seq data, and finally three important genes were identified by expression analysis in the sterile and fertile anthers of thermo-sensitive male sterile wheat. TaPG93 was involved in the pollen development and elongation of pollen tubes, and TaPG87 and TaPG95 played important roles in the separation of pollen grains and the cracking of anthers dehiscence. Conclusions: This study, we performed a thorough analysis of the wheat PG gene family and finally obtained three TaPGs that affect wheat fertility. This will lay a solid foundation for the function exploration of wheat PG gene family and provide new enlightenment for the fertility conversion mechanism of male sterile wheat.

Background

Polygalacturonase (PG, Ec, 3.2.1.15) is a cell wall binding protein that catalyzes the cleavage of α -(1,4)-polygalacturonic acid in pectin. Among numerous enzymes that degrade pectin, PG belongs to the largest family of hydrolases. In 1956, PG was first studied and confirmed to be closely related to fruit softening. Subsequent studies have shown that the main functions of this enzyme is to degrade polygalacturonic acid in the cell wall of fruits into galactose and aldehyde acid, which disintegrates the cell wall structure and leads to fruit softening [1, 2]. Fabi et al found that the overexpression of *cp-PG1* in papaya can promote the softening of pulp [3]. However, with the application of molecular biology technology in PG research, people have a deeper understanding of it. Roongsattham et al identified 14 PG genes during the maturation and shedding of oil palm fruit, all of which were expressed in the detachment of the fruit base at ripening [4]. A PG gene *RDPG1* in *Brassica napus* was expressed mainly in the dehiscence zone of fruit pods and anthers, and also in the detachment zone of floral organs and the nodes of stems and pedicels during the growth of pollen tubes [5]. *Arabidopsis thaliana* PG gene *QRT3* participates in the separation of tetrad microspores during the pollen development process by secreting and degrading pollen mother cell walls around the tetraploid microspores [6]. Bergey [7] and Orozco-Cardenas [8] suggested that injury

induced the expression of PG genes, which produced plant-derived endogenous elicitors, and further activated defense genes *PPO* and *pis* in mesophyll cells. Those proved that members of the PG gene family can be expressed in different tissues and different stages of plant development, in addition to its role in fruit ripening, PGs are also associated with leaf and flower shedding, pod cracking, pollen maturation, pathogen defense, and plant host interaction [9, 10]. Therefore, other functions of PGs on non-fruit plants are also worthy of further study, although its role in affecting fruit ripening has always been the focus of attention.

PGs can be classified into endo-polygalacturonase (endo-PG, Ec, 3.2.1.15), exo-polygalacturonase (exo-PG) and rhamno-galactosidase (oligo-PG) depending on the site of the substrate [11]. According to the characteristic of amino acid sequences, Hadfield et al. classify PGs into three classes of A-C: the differentiation unit A consists of genes expressed in non-pollen tissues, which encode PG genes that lack of a pre-sequence proteins; the differentiation unit B consists of all PG genes encoding a pre-sequence, and the differentiation unit C consists of PG genes expressed in pollen, encoding exo-PGs [12]. Although this classification was determined by the PGs sequence, it also reflected the function differences of PGs, and different subclasses of PG have unique biological functions. However, Park et al. elaborated the division of PG gene family members from algae to terrestrial plants through cluster analysis. 225 PG members from eight species were clustered into six branches from A to F. Class A and B contained PG genes from terrestrial plants, and class E contained PG genes from algae to flowering plants, while C, D, F contained only the PG genes of flowering plants. PGs of Class A and Class B are endo-PG, Class C and Class D PGs are exo-PG, Class E PGs are oligo-PG, while members of Class F cannot be classified into any of properties of PG [11]. The A to F six class PG genes in *Arabidopsis* differ in expression patterns, with some class members tending to be expressed in vegetative tissues, while others tend to be expressed in reproductive organs [13]. Therefore, it is more detailed and convenient to screen functions related genes according to cluster classification rules, which is suitable for PG gene family analysis of most crops.

Based on the clustering classification rules, systematically analysis of the PG gene family have been carried out on *Arabidopsis* and rice [13], soybean [14], apple [15], poplar [16] and other crops, which provide great convenience for function mining of plant PG gene family. However, no analysis of the PG gene family has been reported in wheat. Wheat is a heterologous hexaploid organism with a total of 42 chromosomes, and the research progress was limited due to its large but incomplete genomic information. However, the completion of wheat genome sequencing and sequence release make it possible to explore and study important functional genes of wheat at the genome-wide level [17]. Since the pollen-specific PG genes in tobacco [18] and cotton [19] and *Arabidopsis* [20] have been studied, the study of wheat pollen-specific genes is well-founded and will provide a new perspective for wheat male sterile research.

KTM3315A is a thermo-sensitive male sterile wheat material that with excellent traits and great potential for hybrid production, which our team has always attached great importance to research. In the previous study, we found that KTM3315A showed male sterile under normal autumn sowing conditions in

Shaanxi, China, but it showed fertility can be self-crossed during spring sowing in the same area. By microscopic observation, the binucleate stage was identified as a critical period of fertility conversion, from which the microspore mother cells of the sterile pollens exhibited abnormal shapes such as shrinkage and irregularity [21]. Transcriptome analysis proved that such abortive morphology was closely related to the expression of pectinase [21]. Obviously, it is necessary to study the pollen-specific PG genes that involved in pollen development mechanism through excellent male sterile materials, which can help us to cultivate more stable male sterile materials and accelerate the wheat hybridization breeding process.

In this study, a total of 113 members of wheat PG gene family were identified, and a systematic and comprehensive overview was performed through the analysis of its evolutionary relationships, gene structures, conserved domains, cis-elements and expression patterns. Based on the expression patterns in sterile and fertile anthers, we screened out three *TaPGs* related to pollen development and anther dehiscence. This is both pioneering and a meaningful study in wheat.

Results

Identification and annotation of wheat PG gene family

The Hidden Markov Model (HMM) file of Glycoside hydrolase family 28 (GH28, EC 3.2.1.) and the amino acid sequences of PGs of *Arabidopsis* were aligned with the whole amino acid sequences of wheat, and a total of 113 wheat PGs were finally obtained after strict screening and identification (Additional file 1: Table S1). These 113 wheat PGs were unevenly distributed on 21 chromosomes, and each chromosome had at least one PG. However, there were five PG genes distributed on the A, B and D of chromosomes 2 and 5 respectively, as well as chromosomes of 1D, 4A, and 4B. (Additional file 2: Figure S1). According to their chromosomal location, these 113 wheat PG genes were renamed as *TaPG01-TaPG113*. The 113 *TaPGs* have a CDS length of 762-1617 bp, and an exon number of 2-10. The molecular weight of wheat PG proteins ranged from 26.69-57.99 KDa, with a theoretical isoelectric point of 4.8-10.45, and encoding 253-538 amino acids (Table 1). Accurate identification and reasonable naming are essential for further research in this gene family.

Evolutionary analysis of wheat PG gene family

To understand the evolution pattern of wheat PG, a total of 238 PG proteins from wheat, *Arabidopsis* and rice were used to construct a phylogenetic tree (Fig. 1). The results showed that 238 PG proteins of these three species were clustered into six branches (A-F). Among these six classes, class A had 12 *TaPGs*, and class B to class F had 16, 13, 28, 30, and 14 *TaPGs* in sequence. In addition, there were 39 pairs of paralogous genes in 113 *TaPGs*, of which A class has four pairs of paralogous genes, class B, C and F all had five pairs, while class D and E has nine and eleven pairs of paralogous genes respectively. However, there was only one pair of PG orthologous of rice and wheat (LOC_Os01g33300/*TaPG44*) in the D class. Therefore, it can be speculated that the expansion of the wheat PG family mainly occurred after species differentiation from ancestor.

The duplications of wheat PGs

In order to find out the expansion dynamics and evolution direction of the wheat PG gene family, we investigated its gene duplication events. The duplications of the wheat genome indicated that there were eight pairs of tandem duplications and 87 pairs of segmental duplications between *TaPGs*. The KA/KS values of these eight pairs of tandem duplication *TaPGs* were all less than 1, indicating that they were in the purify selection (Table 2). 93 *TaPGs* involved in segmental duplications, accounting for 82.30% (93/113) of all *TaPGs* and it is 34.58% higher than the probability of segmental duplications of whole wheat genome (number of all genes: 120744, number of segmental duplication genes: 57613, percentage: 47.72%) (Fig. 2, Additional file 3: Table S2). This is a good evidence that the segmental duplications promote the expansion of wheat PG gene family.

Gene structure and conserved motif analysis of wheat PGs

The alignment of amino acid sequences of 113 wheat PGs indicated that most of them contained four intact or tend to be intact conserved domains (Ⅰ 'SPNTDGI', Ⅱ 'GDDC', Ⅲ 'CGPGHGISIGSLG', Ⅳ 'RIK') (Fig. 3). However, some PGs lack a certain domain, for example, in the B class, the amino acid sequences of the conserved domains of *TaPG07* and *TaPG81* were missing and incomplete. The most obvious deletions were shown in class E PGs, just as in the case of apple [15], the III conserved domains of all members of wheat class E PGs were missing. In addition, some of the conserved domains of PGs were complete, but individual amino acids were mutated. Most of the PGs in class E mutated the serine (S) to alanine (A) or threonine (T) in the conserved motif 'SPNTDGIH'. The domain IV was the most conserved region, except for the deletion in *TaPG07* and the mutation of class E PGs, which completely conserved in the remaining *TaPGs*. In summary, the PGs in E class were the most variable members of the family.

MEME tool was used to search for the conserved motifs of 113 wheat PGs, and ten conserved motifs were obtained (Fig. 4, Additional file 4: Figure S2). No PG had all 10 motifs. Wheat PGs of class A and class C had the same five motifs, while all class D wheat PG members had the same nine motifs except for *TaPG92*. It was found that motif 1 and motif 3 both contained conserved domain I 'SPNTDGI' and II 'GDDC' of PGs at the same time, and motif 1 also contained the portion of the conserved domain III 'CGPGHGISIGSLG', while motif 2 and motif 6 contained a conserved domain IV 'RIK'. In addition to the above four conserved motifs, motif 5 and motif 9 were the most conservative because they existed in almost all PGs including group E PGs. The structure of *TaPGs* showed that most of the PG genes had 1-9 introns, among which classes of C and D PGs belonging to exo-PG had relatively shorter gene sequences and fewer introns, and E class PGs belonging to oligo-PG generally had longer intron sequences, while class F PGs generally had more intron numbers (7-9). From the analysis of motifs and gene structures, not only the structural characteristics of each class of PGs can be obtained, but also provided a basis for classification of PG functions.

GO annotation and cis-elements prediction of wheat PGs

It is a very effective means to obtain corresponding gene functions by searching for annotation information in kind of databases and predicting upstream regulatory factors. We obtained the molecular function, biological process and cellular component of 113 *TaPGs* by searching for GO (Gene ontology) annotations. The GO annotations showed that most of the 113 *TaPGs* (68) were active in extracellular region, and all 113 *TaPGs* exerted the hydrolysis and catalytic activity and involved in the primary metabolic and organic substance metabolic process (Fig. 5, Additional file 5: Table S3). Cis-acting elements of *TaPGs* were predicted, and ten of them were analyzed here. They included cis-elements responded for the anaerobic induction (ARE), light (G-box), low-temperature (LTR), also included cis-elements regulated by hormones and transcription factors, such as cis-acting elements involved in the abscisic acid (ABRE), gibberellin (P-Box), auxin (TGA-element), methyl jasmonate (CGTCA-motif, TGACG-motif), and MYB binding site involved in drought-inducibility (MBS), as well as cis-acting regulatory element related to meristem expression(CAT-box). As can be seen from Figure 6, most *TaPGs* (85) may respond to light, 38 *TaPGs* respond to low temperature, 83 *TaPGs* respond to abscisic acid, 20 *TaPGs* respond to gibberellin, and 32 *TaPGs* participate in meristem expression.

Analysis of the expression patterns of *TaPGs*

To predict the relevant functions based on the expression specificity of PG, a heatmap were generated using RNA-seq data from Chinese spring wheat in 15 different developmental stages and tissues (Fig. 7). Obviously, 21, 7, 11 *TaPGs* were significantly up-regulated in the anthesis spikes (spike, reproductive, anthesis), spikes wrapped in flag leaves (spike, reproductive, flag leaf stage), and grains two days post anthesis (grain, reproductive, 2 dpa). These 21 up-regulated *TaPGs* in the anthesis spikes belonged to the D class, except for three from the B class. The 11 up-regulated *TaPGs* in spikes of the flag leaf stage were derived from the B, C, and D classes, and the up-regulated *TaPGs* of the grain belonged to the F class. The results of these preference expressions strongly demonstrated that *TaPGs* of different classifications have a clear division of labor in different tissues. In order to explore *TaPGs* that play an important role in wheat pollen development, we focused on twelve completely spike-specific expressed *TaPGs* (*TaPG09*, *14*, *18*, *28*, *40*, *41*, *49*, *50*, *54*, *87*, *93*, *95*).

Pollen-specific *TaPGs* identified

Real-time quantitative PCR (qRT-PCR) was performed on eight of spike-specific expressed *TaPGs* by sterile and fertile anthers of thermo-sensitive male sterile wheat KTM3315A to understand their expression modes in anthers. The results showed that three *TaPGs* (*TaPG14*, *40*, *41*) that specifically expressed in the spikes of the flag leaf stage showed a decreasing trend with the development of anthers, while the anthesis spike-specific *TaPGs* (*TaPG18*, *28*, *87*, *93*, *95*) were up-regulated during the late anther development (Fig. 8). Just as confirmed by RT-PCR (Additional file 6: Figure S3), these differentially expressed data during anther development supported the fact that these spike-specific *TaPGs* were actually expressed in anthers. Although *TaPG14*, *TaPG40* and *TaPG41* were up-regulated at the uninucleate stage, their role in fertility conversion could be ruled out for they were not significantly differentially expressed between sterile and fertile anthers. *TaPG18*, *TaPG28*, *TaPG87*, *TaPG93*, and

TaPG95 all had the highest expression levels at the trinucleate or binucleate stage in fertile anthers and showed significant differences in expression level with sterile anthers. However, the expression level of *TaPG87* was significantly down-regulated in fertile anthers compared with sterile anthers, while the other four *TaPGs* were opposite. Their non-uniform expression patterns suggested that they had different roles in the development of anthers, and their functions were not redundant even if *TaPG87* and *TaPG95* all belong to class B in this gene family.

By performing an alignment analysis on the above five differentially expressed *TaPGs*, we found that their protein sequences were highly similar to four *Arabidopsis* proteins (AT4G18180, PGA4, ADPG2, ADPG1), respectively. Like *TaPG18* (401aa), the similarity between *TaPG28* (401aa) and AT4G18180 (422a) was also bit-score 364.8, the bit-score of *TaPG93* (294aa) and PGA4 (431aa, AT1G02790) was 263, *TaPG87* (402aa) and ADPG2 (AT2G41850, 433aa) was 399.4, and *TaPG95* (420aa) and ADPG1 (AT3G57510, 431aa) was 401. AT4G18180 belonged to the pectin lyase-like superfamily protein and its function was described as polygalacturonase activity, but its role in anthers has not been determined. However, PGA4 was identified as exo-polygalacturonase and played a role in the depolymerization of pectin during pollen development, pollen grain germination, and pollen tube growth. Its interaction protein included five pectin lyase-like superfamily proteins, two beta-D-xylosidase, two alpha-L-arabinofuranosidase, and a glycosyl hydrolase 9A1 involved in cellulose biosynthesis. ADPG2 was identified as a polygalacturonase involved in cell separation in the final stages of pod shatter, in anther dehiscence and in floral organ abscission, and part of its interaction proteins have the function of organ shedding. ADPG1 was a polygalacturonase protein involved in silique and anther dehiscence, and most of its interaction proteins belonged to pectin lyase-like superfamily protein (Additional file 7: Table S4). Therefore, by aligning with the more well-studied model plant *Arabidopsis* to obtain sequence-similar *Arabidopsis* PG genes and predicting functions of the corresponding *TaPGs*, three *TaPGs* worth studying have broken into our field of vision. *TaPG93* was significantly up-regulated during the binucleate stage and was supposed to be involved in pollen development and pollen tube growth, while *TaPG87* and *TaPG95*, which were up-regulated in the trinucleate stage, played an essential role in pollen separation and anther dehiscence.

Discussion

Polygalacturonase, a glucosyl hydrolase family 28, also known as pectic enzyme or pectin depolymerase. It is a gene family that is closely related to plant development, and playing roles by expressing them in different time and space, or by encoding different hydrolysis patterns or binding different substrates. In recent years, the development of genome sequencing has made it possible to systematically study the evolution, localization, structural and expression of plant PG genes. 66 PG genes were identified in *Arabidopsis* [12], 46 PG genes were identified in rice [13], 62 and 53 were identified in watermelon and cucumber [22], and 75, 85, 112, and 99 in *Populus*, apple, soybean, and *Brassica rapa*, respectively [14-16, 23]. In this study, we identified 113 wheat PG genes and demonstrated that PG gene family plays a complex role in higher plant wheat. The existence of genome-wide repeats and other repetitive events have led to the generation of many repetitive genes in the PG gene family during evolution [13]. Function

redundancy and function differentiation may occur among numerous PG members due to the high degree of similarity of sequences [24]. From the duplication analysis of *TaPGs*, it can be seen there was higher synteny probability occurred between ABD sub-genomic in the same chromosome groups of wheat than that between different chromosome groups. There were eight pairs of tandem duplications and 87 pairs of segmental duplications in wheat PG gene family, and the proportion of segmental duplications of *TaPGs* was much higher than that of whole genomes. Therefore, we speculated that the main driving force for the expansion of wheat PG gene family was genome replication and segmental duplications.

Typical PGs have four conserved domains in plants and fungi: the amino acid sequences of domain I and domain II are 'SPNTDGI' and 'GDDC', respectively, and the three aspartic acid (D) of them may be components of the catalytic site [25]; the sequence of domain III is 'CGPGHGISIGSLG', in which the histidine residue (H) is considered to be involved in the catalytic reaction [26]; the sequence of domain IV is 'RIK' with a positive charge, which may be involved in ionic interaction with the carboxy terminus of the substrate [27]. In general, a gene encoding a protein having the above four conserved domains is identified to belong PG gene family, but it is worth noting that the conservation of domain III is relatively poor [12]. This is consistent with our research. All *TaPGs* in class E did not have a conserved domain III, and only five of them kept completely conserved for remaining three domains, while others undergone at least one amino acid variation. Compared to other classes of PGs, class E PGs are widely present in algae to flowering plants, so they have experienced more natural selection pressures and more variation during the long evolutionary process.

PG acts as a downstream enzyme of the metabolic pathway, its expression and activity are regulated by other genes, proteins, plant hormones. In *Arabidopsis*, transcription factors BHLH, RPK2 AtDOF4.7 and AtC3H14 have been identified to regulate PG gene expression [20, 28-30]. In our research, we found that MYB was also involved in the regulation of PG. For the regulation of PGs by hormones, it has been studied in many species. Sun et al. inhibited the expression of SINCED1, a key enzyme in the synthesis of abscisic acid (ABA) in tomato, resulting in down-regulation of tomato PG gene [31]. In the transcriptome analysis of the ripening and shedding areas of oil palm fruit, it was shown that the expression abundance of *EgPG4* increased by 700-5000 times in the base of the fruit when treated with ethylene [4]. Ziosi et al. applied MeJA to nectarine fruits at different developmental stages, which down-regulated the expression of fruit ripening genes including PGs, thereby delaying fruit ripening [32]. In *Arabidopsis*, When treated with jasmonate (JA), the expression levels of *ADPG1*, *ADPG2*, and *QRT2* in the anthers were increased by about 10 times [20]. Although the hormones that regulate fruit ripening and shedding ultimately work by positively regulating PGs, the hormones that regulate PGs obtained by cis-element analysis in this study should behave as they were in *Arabidopsis*, which affected anther development and cracking.

In angiosperms, the main component of pollen inner wall is pectin, and the pollen tube wall is an extension of the inner wall of pollen[33]. Castro et al. used the monoclonal antibody LM5 immunolocalization technique revealed the presence of pectin in the pollen tube and pollen grains of olives, and found that the most abundant pectin was distributed in the three germinal holes [34, 35]. Along with the presence of pectin, many pectin degrading enzymes have been found in plant anthers,

including pectin lyase, polygalacturonase, polymethylgalacturonase, pectin methylesterase [36-39]. As early as the 1990s, PG was found to be widespread in the late stages of plant anther development [18, 36, 40, 41], and the exploration of their action mechanism has been very frequent in recent years. *BcMF2* was specifically expressed in the tapetum and pollen grains in the late tetrad, however, in the inhibited mutants *bcmf2*, the inner wall of the mature pollen grains was in a disordered state, the pollen tube failed to germinate normally, and the fertility of the plant was reduced [42]. *BcMF9* was also expressed in the tapetum and microspores of late stage of pollen development, which affected the development of pollen and pollen tubes by affecting the formation of inner and outer walls [43]. In addition, in Chinese cabbage, *BcMF6*, *BcMF16*, and *BcMF17* have also been identified as PG genes that play an important role in pollen development [44-46]. In *Arabidopsis*, three endo-PGs, including ADPG1 and ADPG2 and QRT2, were critical for pollen grains separation and contributed to anther dehiscence, but the overexpressing mutants of *QRT2* caused male sterility [20].

In this study, by sequence similarity search, the protein sequences of *TaPG87* and *TaPG95* were found to be highly similar to the ADPG2 and ADPG1, respectively. From the qRT-PCR results, we found that they all expressed in the late stage (trinucleate) of fertile anthers just as many of the pollen-specific PGs that have been studied above, so we speculate that they are responsible for the separation of pollen grains and the cracking of anthers, like *ADPG1* and *ADPG2* in *Arabidopsis*. Although the down-regulated expression of *TaPG87* in fertile anthers is not as expected, the result have been repeatedly verified by RT-PCR (Additional file 6: Figure S3) and qRT-PCR. This suggested that different expression levels of *TaPGs* may have different effects on the regulation of anther development, just as moderate *QRT2* promotes anther development but overexpression can lead to male sterile. Therefore, the effect of precise regulation of *TaPG87* on wheat fertility deserves further study. The interacting proteins of PGA4 were several proteases that hydrolyze substrates, including β -D-xylosidase, α -L-arabinofuranosidase, glycosyl hydrolase and pectin lyase-like superfamily proteins. These enzymes have been shown to play important roles such as degrade the callosic wall surrounding the microspores and were closely related to the male fertility of plants [47-51]. The binucleate stage is a critical period for fertility conversion of thermosensitive male sterile wheat KTM3315A, which coincides with the highest expression stage of *TaPG93* in fertile anthers. Therefore, combined with its similar protein PGA4 and interaction proteins as well as typical expression pattern, we identified *TaPG93* as a key gene affecting pollen development and pollen tube growth, and also a crucial gene in KTM3315A during the fertility conversion process. Therefore, based on this experiment, further subcellular localization and transgene verification of *TaPG93* will be of great significance and our next task..

Conclusions

PG is a pectin hydrolase closely related to the development of plants involved in complex functions. It is important to understand pollen-specific PGs in wheat, which is extremely valuable for exploring pollen development mechanisms and the utilization of male sterile and heterosis. To systematically study wheat PG gene family, we identified a total of 113 PGs that were unevenly distributed on 21 chromosomes using the information of newly published wheat reference genomic. Based on the physical location, they were

renamed as *TaPG01-TaPG113*. According to the phylogenetic tree constructed with *Arabidopsis* and rice, 113 *TaPGs* were divided into six branches of A-G, of which class E PGs had the lowest conservation and no III conserved domains. Duplications analysis revealed that one of the main drivers of wheat PG gene family expansion was segmental duplication. The analysis of cis-elements revealed the response of *TaPGs* to light, anaerobic, low temperature and other environments, and it also provided a new basis for studying the regulation of wheat PG by hormones. Through expression analysis, twelve spike-specific *TaPGs* were identified. After further analysis, these spike-specific genes were actually expressed in anther and three of them were predicted as important roles in pollen development and anther dehiscence. This provided an important theoretical basis for cultivating excellent male sterile materials.

Methods

Plant Materials

In this experiment, we used a wheat material KTM3315A selected by our research team in 2001 at the Northwest A&F University of Yangling, Shaanxi, China. KTM3315A is a thermo-sensitive male sterile wheat material that our research group has been focusing on because of its strong agronomic traits and stable fertility performance. It exhibits sterile in low temperature conditions and fertile under conditions of high temperature, so it can both achieve self-crossing and be an advantageous materials for heterosis utilization [21]. Each 10 pots of wheat KTM3315A were placed in two artificial climate incubators with parameters set to 14 h light (day) and 10 h dark (night), and the day/night temperatures were 17 °C/15 °C (sterile condition) and 22 °C/20 °C (fertile condition), respectively. A total of 20 pots of KTM3315A were treated at different temperatures until grouting was completed. The roots, stems, leaves and anthers from both sterile and fertile plants in three stages (uninucleate, binucleate and trinucleate) were saved in the -80 °C refrigerator, as well as freshly grouted grains of fertile plants.

Identification of wheat PGs gene family

The whole protein sequences of wheat and the HMM file of GH28 were downloaded from the Ensembl plant (http://plants.ensembl.org/Triticum_aestivum/Info/Index) [52] and Pfam databases (<http://pfam.xfam.org/family/PF00295>) [53], respectively. A wheat-specific HMM file was constructed using the results of the wheat protein sequences alignment with the GH28 HMM file with the e-value was less than 1e-20. Then, The newly constructed wheat-specific HMM file was used again to align with the wheat protein sequences, and the results with an e-value of less than 0.01 were identified as candidate wheat PGs. To avoid omissions, we aligned whole wheat protein sequences with PGs sequences of *Arabidopsis* that downloaded from phytozome [13]. The above two strategies obtained all candidate wheat PGs, and the final wheat PGs were identified after screening by CD-search (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) [54], SMART (<http://smart.embl.de/>) [55] and pfam (<http://pfam.xfam.org/>) [53]. Their genetic location information were obtained from reference genome annotation. The amino acid length of PG proteins, molecular weight and isoelectric point were predicted by Expsy (<https://www.expasy.org/structural/bioinformatics>).

Constructing phylogenetic tree of wheat PGs, and analysis of gene duplications

In order to sort out the evolutionary relationship of wheat PGs, the amino acid sequences of 66 *Arabidopsis* PGs, 59 rice PGs and 114 wheat PGs were performed for clustalw sequence alignment and phylogenetic tree construction by MAGE. The phylogenetic tree was constructed by using the Neighbor-Joining (NJ) method with parameters were set as repeated with bootstrap 1000 times. Tandem duplications and segmental duplications of the wheat PG gene family were obtained by Mcscanx [56] and the ratios of the nonsynonymous substitution rate to the synonymous substitution rate (KA/KS) of tandem duplication PGs were calculated by the KaKs_Calculator2.0 [57].

Analysis of gene structure and conserved domain of wheat PGs

From multiple sequence alignment analysis of conserved regions, we obtained four conserved domains of the PG gene family and visualized them using GeneDoc software. Using MEME, 10 conserved motifs with lengths of 6-50 were obtained. Combined with the wheat genome annotation information, the genetic structure and conserved motifs of wheat PGs were visualized by TB-tools [58].

GO annotation, cis-element and function predictions of *TaPGs*

To understand the molecular functions, biological Processes and cellular components of wheat PGs, the GO annotation of wheat PGs were obtained from ensembl plant. It is helpful to understand the upstream regulatory factors of PG by analyzing cis-elements. Therefore, the 1500 bp sequences of upstream of the *TaPGs* promoter were used to predict the cis-elements of PGs via plantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [59]. To predict the function of *TaPGs*, STRING was used to obtain *Arabidopsis* PGs that with similar sequences to wheat PGs, and their functions and interactions [60].

Expression analysis of *TaPGs*

In order to screen genes based on expression preference, RNA-seq data from 15 different tissues (root, stem, leaf, spike, grain) and developmental stages (seedling, vegetative, reproductive) of Chinese spring were used to analyze the expression profile of wheat PG genes [61]. To further verify whether spike-specific *TaPGs* are associated with anther development and fertility conversion, a relative quantitative analysis of sterile and fertile anthers was performed on qRT-PCR in three stages (uninucleate, binucleate, trinucleate). Total RNA was extracted using TRIgene reagent (GeneStar), and reverse transcription of CDNA and RT-PCR were performed by [StarScript II First-strand cDNA Synthesis Kit-II](#) (GeneStar) and 2×RealStar Green Fast Mixture (GeneStar), respectively. All technical procedures were referred to the manufacturer's instructions. We used the wheat Actin gene as an internal reference and calculated the relative expression level of each gene in sterile and fertile plants by $2^{-\Delta\Delta Ct}$ based on the Ct (cycle threshold) value obtained from the Applied Biosystems 7500 Real-Time PCR System.

Abbreviations

ABA: Absciscic acid; Ct: cycle threshold; dpa: days post anthesis; endo-PG: endo-polygalacturonase ; exo-PG: exo-polygalacturonase; GH28: Glucosyl hydrolase family 28; GO: Gene ontology; HMM: Hidden Markov Model ; JA: jasmonate; Ka: nonsynonymous substitution rate; Ks: synonymous substitution rate; oligo-PG: rhamno-galactosidase; PG: Polygalacturonase

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request

Competing interest

The authors declare that they have no competing interests

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Authors' contributions

XS and LZ designed the research. JY performed the experiments. JY, XY, WL and QL analyzed the data. JY wrote the manuscript with contributions from all the authors. XS critically revised the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1. Annotation of 113 *TaPGs*.

Name	ID	Start	End	CDS Length	Exon	aa	MW	pl
		locus	locus					
<i>TaPG01</i>	TraesCS1A02G124300.1	146944161	146946921	1302	5	433	47.46	9.44
<i>TaPG02</i>	TraesCS1A02G311100.1	502804558	502806739	1455	3	484	50.95	6.07
<i>TaPG03</i>	TraesCS1A02G364800.1	544389268	544392159	1218	8	405	42.67	7.4
<i>TaPG04</i>	TraesCS1A02G418000.1	575594342	575597857	1533	7	510	54.38	6.19
<i>TaPG05</i>	TraesCS1B02G142700.1	192274006	192276920	1323	6	440	48.02	9.44
<i>TaPG06</i>	TraesCS1B02G322500.1	547522874	547524954	1455	3	484	51.04	6.07
<i>TaPG07</i>	TraesCS1B02G382200.1	614499777	614502412	762	6	253	26.69	6.07
<i>TaPG08</i>	TraesCS1B02G448100.1	667491123	667494420	1593	7	530	56.51	6.72
<i>TaPG09</i>	TraesCS1D02G066400.1	47934766	47936019	1146	4	381	41.84	5.96
<i>TaPG10</i>	TraesCS1D02G129600.1	143256701	143260129	945	5	314	34.55	9.38
<i>TaPG11</i>	TraesCS1D02G311000.1	407159790	407161900	1440	3	437	46.90	8.84
<i>TaPG12</i>	TraesCS1D02G370300.1	448786644	448790864	1011	7	420	44.26	6.03
<i>TaPG13</i>	TraesCS1D02G425600.1	479227885	479231447	1584	7	420	44.26	5.85
<i>TaPG14</i>	TraesCS2A02G361400.1	605310908	605312618	1179	4	473	50.20	6.41
<i>TaPG15</i>	TraesCS2A02G405100.1	661081507	661084048	1254	9	420	45.39	9.03
<i>TaPG16</i>	TraesCS2A02G405300.1	661151264	661153827	1218	9	404	42.36	6.31
<i>TaPG17</i>	TraesCS2A02G468400.1	712314740	712318931	1248	7	485	52.10	6.43
<i>TaPG18</i>	TraesCS2A02G469000.1	712706310	712708324	945	5	488	51.92	5.15
<i>TaPG19</i>	TraesCS2B02G423200.1	608539784	608542245	1278	9	415	43.80	7.39
<i>TaPG20</i>	TraesCS2B02G423300.1	608666849	608669361	1206	9	463	49.72	9.05
<i>TaPG21</i>	TraesCS2B02G423400.1	608801855	608804223	1056	9	479	50.64	6.07
<i>TaPG22</i>	TraesCS2B02G491300.1	689713615	689715922	1248	5	414	43.58	6.16
<i>TaPG23</i>	TraesCS2B02G491900.1	690210442	690212803	1206	4	414	43.60	6.16
<i>TaPG24</i>	TraesCS2D02G402000.1	516862211	516864619	1287	9	526	55.50	6.63
<i>TaPG25</i>	TraesCS2D02G402100.1	516924323	516926882	1218	9	528	56.24	5.6
<i>TaPG26</i>	TraesCS2D02G402200.1	517037413	517046181	1176	8	336	35.90	6.87
<i>TaPG27</i>	TraesCS2D02G468500.1	573848903	573853711	1248	7	527	56.32	6.72
<i>TaPG28</i>	TraesCS2D02G469100.1	574345359	574347703	1206	4	392	41.07	8.87
<i>TaPG29</i>	TraesCS3A02G129400.1	106660848	106663530	1233	10	417	43.40	4.8
<i>TaPG30</i>	TraesCS3A02G129500.1	106676627	106678931	1224	10	405	42.76	9.1
<i>TaPG31</i>	TraesCS3A02G225800.1	423487241	423491329	1617	7	415	45.01	7.53
<i>TaPG32</i>	TraesCS3A02G393600.1	641557983	641559792	1365	3	401	43.48	7.56
<i>TaPG33</i>	TraesCS3A02G401500.1	647463244	647465368	1212	4	425	44.24	5.21
<i>TaPG34</i>	TraesCS3A02G505300.1	727180691	727182147	1143	4	401	42.55	9.01
<i>TaPG35</i>	TraesCS3B02G148500.1	139778020	139780581	1206	10	351	37.58	8.82
<i>TaPG36</i>	TraesCS3B02G148600.1	139831889	139834882	1239	10	415	44.94	7.86
<i>TaPG37</i>	TraesCS3B02G253000.1	406459430	406464343	1353	6	401	43.49	7.55
<i>TaPG38</i>	TraesCS3B02G258600.1	416275603	416280269	1611	7	428	44.27	4.82
<i>TaPG39</i>	TraesCS3B02G434400.1	672851360	672853399	1221	4	405	42.70	8.78
<i>TaPG40</i>	TraesCS3B02G572500.1	803511461	803513157	1182	4	391	41.59	8.77
<i>TaPG41</i>	TraesCS3B02G573100.1	804111639	804113487	1179	4	415	44.78	7.86
<i>TaPG42</i>	TraesCS3D02G130300.1	90372659	90375261	1206	10	401	43.52	7.55
<i>TaPG43</i>	TraesCS3D02G130400.1	90379298	90381821	1239	10	410	44.23	8.48
<i>TaPG44</i>	TraesCS3D02G202000.1	243323233	243328008	1284	6	407	43.59	6.93
<i>TaPG45</i>	TraesCS3D02G223900.1	305452677	305456655	1611	7	538	57.64	8.89
<i>TaPG46</i>	TraesCS3D02G230000.1	313310405	313314040	1353	5	454	49.90	7.61
<i>TaPG47</i>	TraesCS3D02G387300.1	502125506	502127350	1365	3	403	42.62	7.98
<i>TaPG48</i>	TraesCS3D02G396300.1	511330504	511332483	1221	4	380	40.19	8.83
<i>TaPG49</i>	TraesCS3D02G512500.1	596993603	596995622	1182	4	401	43.34	7.52
<i>TaPG50</i>	TraesCS3D02G512900.1	597264600	597265932	1032	4	412	44.13	7.18
<i>TaPG51</i>	TraesCS4A02G066700.1	63824843	63827423	1302	9	450	47.83	7.61

TaPG52	TraesCS4A02G168800.1	423154931	423158789	1374	6	536	57.53	8.89
TaPG53	TraesCS4A02G229600.1	538839203	538840998	1206	4	406	43.05	7.93
TaPG54	TraesCS4A02G229700.1	538904932	538906842	1188	4	393	41.83	9.21
TaPG55	TraesCS4A02G358700.1	631920845	631925008	1350	7	392	41.05	8.87
TaPG56	TraesCS4B02G086400.1	86359231	86361142	1185	4	401	43.16	8.35
TaPG57	TraesCS4B02G086500.1	86465040	86466774	1206	4	412	44.06	6.84
TaPG58	TraesCS4B02G144700.1	195460952	195464972	1365	6	427	45.49	5.8
TaPG59	TraesCS4B02G244300.1	505543818	505546374	1302	9	536	57.41	8.94
TaPG60	TraesCS4B02G344700.1	638122183	638125126	1461	5	450	47.63	7.57
TaPG61	TraesCS4D02G146700.1	136183828	136187858	1374	6	454	49.97	8.29
TaPG62	TraesCS4D02G243600.1	406231167	406233698	1302	9	406	43.04	7.98
TaPG63	TraesCS5A02G090800.1	123708094	123713351	1350	6	393	41.46	8.96
TaPG64	TraesCS5A02G212900.1	428148072	428151619	1596	5	434	36.83	10.45
TaPG65	TraesCS5A02G264400.1	476667366	476670027	1359	3	433	46.39	5.39
TaPG66	TraesCS5A02G468900.1	646057598	646059720	1251	4	457	49.53	5.17
TaPG67	TraesCS5A02G513600.1	677160384	677163913	1356	6	401	41.44	8.15
TaPG68	TraesCS5B02G097100.1	128215578	128219271	1332	6	395	41.50	9.02
TaPG69	TraesCS5B02G210900.1	381717109	381720627	1596	5	449	48.49	5.71
TaPG70	TraesCS5B02G263700.1	448130267	448132946	1374	3	394	41.19	8.91
TaPG71	TraesCS5B02G481500.1	654365679	654367442	1323	3	401	41.56	8.53
TaPG72	TraesCS5B02G513100.1	678335575	678339587	1431	7	454	49.43	5.38
TaPG73	TraesCS5D02G102900.1	116170170	116175261	1350	6	433	46.28	5.38
TaPG74	TraesCS5D02G219200.1	328436236	328439556	1596	5	486	52.83	5.66
TaPG75	TraesCS5D02G271900.1	375309726	375312492	1374	3	457	49.52	5.17
TaPG76	TraesCS5D02G481800.1	519544686	519546837	1200	4	433	46.23	5.38
TaPG77	TraesCS5D02G513600.1	537671501	537675675	1596	6	449	48.35	5.14
TaPG78	TraesCS6A02G084500.1	53077600	53082399	1578	2	531	57.96	9.55
TaPG79	TraesCS6A02G159000.1	150980807	150982536	1251	2	452	49.48	6.51
TaPG80	TraesCS6A02G340000.1	573405382	573408916	1488	5	416	44.40	8.85
TaPG81	TraesCS6B02G118000.1	112897663	112903149	1500	3	451	48.79	5.72
TaPG82	TraesCS6B02G192300.1	226395703	226397460	1275	2	443	47.75	4.9
TaPG83	TraesCS6B02G371200.1	645351055	645353904	1296	5	531	57.99	9.48
TaPG84	TraesCS6D02G153500.1	127379138	127380712	1251	2	457	49.80	6.75
TaPG85	TraesCS6D02G320600.1	428641253	428644718	1488	6	440	47.16	8.95
TaPG86	TraesCS7A02G019400.1	7936758	7939018	1566	3	476	51.29	5.63
TaPG87	TraesCS7A02G162500.1	118462549	118464330	1263	6	449	48.20	501
TaPG88	TraesCS7A02G270000.1	275971899	275973610	1218	3	531	57.93	9.55
TaPG89	TraesCS7A02G323800.1	470999122	471003850	1038	6	457	49.83	6.79
TaPG90	TraesCS7A02G330000.1	482216406	482219390	1467	6	399	42.62	8.86
TaPG91	TraesCS7A02G404700.1	586691986	586693761	1263	3	531	57.36	6.7
TaPG92	TraesCS7A02G404800.1	587364624	587366468	963	4	525	55.78	5.26
TaPG93	TraesCS7A02G404900.1	587518498	587520054	885	3	416	43.83	8.65
TaPG94	TraesCS7A02G405000.1	588738598	588740487	1263	3	495	54.24	8.82
TaPG95	TraesCS7B02G067800.1	73134816	73136777	1263	6	499	53.04	5.73
TaPG96	TraesCS7B02G168200.1	234912943	234914373	1320	4	424	44.55	8.62
TaPG97	TraesCS7B02G224700.1	424565499	424569003	1458	5	431	46.71	8.55
TaPG98	TraesCS7B02G230600.1	433062559	433065753	1464	6	416	43.74	8.64
TaPG99	TraesCS7B02G304100.1	544881057	544882860	1263	3	495	54.31	9.08
TaPG100	TraesCS7B02G304200.1	544911423	544913191	1314	4	521	55.61	6.7
TaPG101	TraesCS7B02G304400.1	544982183	544983951	1263	3	420	45.31	8.85
TaPG102	TraesCS7B02G304500.1	545973515	545975328	1263	3	405	42.40	6.45
TaPG103	TraesCS7D02G016300.1	7171203	7173625	1422	3	345	38.25	5.57
TaPG104	TraesCS7D02G164200.1	113446046	113448164	1263	6	488	51.86	5.15

<i>TaPG105</i>	TraesCS7D02G270500.1	256219076	256220499	1215	3	420	44.12	8.21
<i>TaPG106</i>	TraesCS7D02G320900.1	411009415	411012798	1458	5	320	33.59	8.66
<i>TaPG107</i>	TraesCS7D02G326800.1	417356133	417359468	1467	6	294	30.79	6.81
<i>TaPG108</i>	TraesCS7D02G397900.1	513311905	513313583	1248	3	420	44.12	8.04
<i>TaPG109</i>	TraesCS7D02G398100.1	514074452	514076294	1392	4	420	45.48	8.91
<i>TaPG110</i>	TraesCS7D02G398900.1	515219721	515221824	1245	3	439	46.63	8.32
<i>TaPG111</i>	TraesCS7D02G399000.1	515235925	515238032	1245	3	485	51.98	6.35
<i>TaPG112</i>	TraesCSU02G066000.1	51233915	51237421	1581	2	487	51.75	5.07
<i>TaPG113</i>	TraesCSU02G113500.1	97473524	97479283	1587	2	420	44.23	6.26

Start locus and End locus represent the start and end positions of the gene on the chromosome, respectively. CDS Length represents the length of the coding sequence of the corresponding gene, in bp. Exon: number of exons of the gene; aa: length of amino acid; MW: molecular mass; pI: isoelectric point.

Table 2. Ka/Ks values of tandem duplication *TaPGs*.

Name	Transcript ID	Ka	Ks	Ka/Ks
TaPG19&TaPG20	TraesCS2B02G423200.1&TraesCS2B02G423300.1	0.978963	1.05556	0.927433
TaPG20&TaPG21	TraesCS2B02G423300.1&TraesCS2B02G423400.1	0.0670902	0.274587	0.244332
TaPG24&TaPG25	TraesCS2D02G402000.1&TraesCS2D02G402100.1	0.960948	1.11393	0.862667
TaPG35&TaPG36	TraesCS3B02G148500.1&TraesCS3B02G148600.1	0.12591	0.337925	0.372598
TaPG42&TaPG43	TraesCS3D02G130300.1&TraesCS3D02G130400.1	0.130337	0.337546	0.386131
TaPG93&TaPG94	TraesCS7A02G404900.1&TraesCS7A02G405000.1	0.0106235	0.0757237	0.140293
TaPG101&TaPG102	TraesCS7B02G304400.1&TraesCS7B02G304500.1	0.00176716	0.0581157	0.0304077
TaPG110&TaPG111	TraesCS7D02G398900.1&TraesCS7D02G399000.1	0.00367189	0.0136337	0.269325

Ka: non-synonymous mutation frequency; Ks: synonymous mutation frequency; Ka/Ks: ratio of non-synonymous mutation rate to synonymous mutation rate. This ratio can be used to determine if there is a selective pressure on this protein-coding gene.

Additional Files

Additional file 1: Table S1. Amino acid sequences of 113 wheat PGs.

Additional file 2: Figure S1. Position distribution of 113 *TaPGs* on chromosomes.

Additional file 3: Table S2. Position information of wheat PG genes involved in segmental duplications.

Additional file 4: Figure S2. Ten motif sequences of wheat PGs.

Additional file 5: Table S3. Details of the GO annotation of the *TaPGs*.

Additional file 6: Figure S3. RT-PCR verification.

Additional file 7: Table S4. Gene annotations and interaction annotations of *Arabidopsis* proteins that with similar sequences to the important *TaPGs*.

Figures

Figure 1

Phylogenetic tree of *Arabidopsis thaliana*, rice and wheat PGs. The shades of different colors on the tree are used to distinguish between different branches, and the letters A-F indicate the classification of the PG gene family.

Figure 2

The synteny relationships of the wheat PGs. The gray lines in the middle represent the synteny of the whole wheat genome, and the orange lines represent the synteny between the PGs.

Figure 3

Amino acid sequences of four conserved domains of wheat PGs. The top notes are the conserved sequences of the four conserved domains in plant PG gene family, and the right side annotation is the different classification of PGs obtained from the phylogenetic tree.

Figure 4

The gene structures of TaPGs and the conserved motifs of wheat PG proteins. The three parts from left to right are the phylogenetic tree of wheat PG gene family, the distribution of CDS and introns on TaPGs, the motifs distribution on the amino acid sequences of PGs. The scales at the bottom represents the genes length, and the numbers on the motifs represent the name of the motifs.

Figure 5

The GO annotations for TaPGs. The red, yellow and green bars represent the cellular components, molecular functions and biological processes of the TaPGs, respectively. The numbers at the end of the bars represent the number of wheat PG genes enriched to the corresponding annotation.

Figure 6

Distribution of cis-elements of TaPGs. The details of the cis-elements are represented by different color legends at the bottom.

Figure 7

Heatmap of TaPGs in 15 different developmental stages and tissues.

Figure 8

qRT-PCR results of 10 TaPGs.

Supplementary Files

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